immunization markedly enhance the degree of contact sensitivity in sensitized animals and reverse tolerance in unresponsive animals [20–22].

In the present study, we observed that the unresponsiveness obtained by topical application of DNCB on PUVA-treated skin was also reversed by intraperitoneal administration of cyclophosphamide. Consequently it can be assumed that the tolerance in these animals is also a specific immunologic unresponsiveness mediated by suppressor cells. Recently we confirmed in mice that the unresponsiveness can be transferred to syngeneic recipients using spleen cells and that the suppression by spleen cells was abrogated by antibody to T cell-specific antigen (Thy 1.2) and complement treatment (unpublished data).

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# Activation of Complement by *Pityrosporum orbiculare*

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**The ability to activate complement in human serum was evaluated for the two yeast-like organisms *Pityrosporum orbiculare*, the presumed etiologic agent of tinea versicolor, and *Candida albicans*. Complement activation was measured by: (a) using inhibition of rabbit red blood cell lysis by human serum after incubation with the organisms, and (b) quantitation of the amount of C3 deposited on the surface of the yeast by an enzyme-linked immunoabsorbent assay. It was found that both organisms had approximately equal ability to activate complement in normal serum or serum having only the alternative pathway intact, even though extracts of *C. albicans* contained significantly greater amounts of both carbohydrate and antigenic material capable of combining with the antibody present in normal human serum. The marked difference in inflammation in the cutaneous lesions produced by these two organisms does not appear to be related to their complement-activating ability and is more likely due to some other factor such as differences in invasiveness or in ability to elicit other immunologic reactions.**

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Abbreviations:
EGTA: ethylene glycol-*bis*-(beta-amino ethyl ether)-N,N′-tetra-acetic acid
ELISA: enzyme-linked immunoabsorbent assay
PBS: phosphate-buffered saline
RBC: red blood cells

Tinea versicolor is a superficial fungal infection that evokes very little inflammatory response by the host. In contrast, superficial cutaneous infections with *Candida albicans* are highly inflammatory, producing neutrophil-containing epidermal microabscesses in acute infections [1] and marked dermal mononuclear cell infiltration in chronic infections [2]. Complement activation via the alternative pathway has been implicated in the production of the former type of inflammatory reaction to cutaneous *Candida* infections [3]. However, the inflammation produced in another type of superficial mycosis, i.e., experimental cutaneous *Trichophyton mentagrophytes* infection, appears to be due mainly to cell-mediated immunity [4]. In an attempt to elucidate the differences in capacity to produce inflammation of *Candida* and *Pityrosporum orbiculare* (the apparent etiologic agent of tinea versicolor), we have previously compared the antigenicity of the two organisms in lymphocyte transformation tests. These experiments demonstrated that *Candida* was significantly more antigenic than was *Pityrosporum* in this system [5].

Complement activation may be involved in microabscess formation, and *Candida* infections are associated with this type of acute infiltrate whereas infections caused by *Pityrosporum* are not. Therefore, the present study was designed to compare complement activation by the two organisms. *Pityrosporum* is a yeast-like organism that has an absolute growth requirement for added lipids in vitro. In previous studies, we have found that most of the yeast grown in a 2-phase system will become physically associated with the oil [5]. Therefore, this organism's surface might be considerably different from that of *Candida* and thus less able to activate complement and elicit acute inflammation.

## MATERIALS AND METHODS

### *Organisms*

Five isolates of *P. orbiculare* were obtained from skin scrapings of tinea versicolor lesions by culture on mycosel agar (BBL, Div. Becton, Dickinson, and Co., Cockeysville, Maryland) overlaid with sterile olive oil. Subcultures were made from emerging colonies and identified as *P. orbiculare* by morphologic appearance and a strict requirement for added lipid. All 5 isolates grew preferentially at the oil–water interface of broth cultures, although 2 also grew in the water phase and could be centrifuged free of oil if 0.1% Tween 80 were added to the medium.

Five isolates of *C. albicans* were obtained from clinical isolates and maintained by repeated passage on Sabouraud's agar. Both organisms were grown in bulk in AOAC synthetic broth (Difco Laboratories, Detroit, Michigan) containing 1% dextrose, chloramphenicol (20 μg/ml), cycloheximide (250 μg/ml), 0.1% Tween 80, and overlaid with sterile olive oil. At the end of the culture period (14 days for *P. orbiculare* and 5 days for *C. albicans*), the oil and water phases were separated by centrifugation and the organisms in the water phase washed 3 times in 0.9% NaCl. The quantity of organisms present was expressed as either ml of packed cells or as dry weight determined after lyophilization of the yeast suspensions. These two methods were used in case one or the other alone might have given an inaccurate estimate of the quantity of organisms present.

### *Preparation of Extracts*

Extracts of the two organisms were prepared as described previously [5] using either the ether extraction method or sonication. Protein concentrations of the extracts were determined by the Lowry technique and carbohydrates by the acid-phenol method [6].

### *Measurement of Complement Activation Using Hemolysis of Rabbit Red Blood Cells (RBC)*

Human sera were incubated with known quantities of yeast or yeast extracts and then evaluated for their ability to hemolyze rabbit RBC by the alternative pathway using a modification of the method of Platts-Mills and Ishizaka [7]. In brief, the yeast or yeast extracts were incubated for 30 min at 37°C with normal human serum, human serum with $5 \times 10^{-3}$ M ethylene glycol-*bis*-(beta-amino ethyl ether)-N,N'-tetraacetic acid (EGTA) containing $5 \times 10^{-3}$ M of $MgCl_2$, or C2-deficient human serum (kindly provided by Dr. Susan Koethe, Medical College of Wisconsin). The yeasts were removed by centrifugation, and the serum was then incubated at a 1:5 dilution with rabbit RBC that had been obtained in heparin and then washed and suspended to approximately $1.5 \times 10^8$ cells/ml of gelatin-veronal-saline at pH 7.6 containing $5 \times 10^{-3}$ M EGTA and $5 \times 10^{-3}$ M $MgCl_2$. The incubation was allowed to proceed for 2 hr at 37°C, after which time 2.5 ml of $10^{-2}$ M EGTA in saline was added.

The tubes were then centrifuged and the resulting supernatants read at 413 nm in a spectrophotometer. The results were compared to those from samples in which 100% RBC lysis was obtained with distilled water. Results were expressed as percent inhibition of hemolysis as a function of either the dry weight or packed volume of organisms employed. The amount required to produce 20% or 50% inhibition of hemolysis was obtained using a series of dilutions of the yeast suspension.

### *Measurement of Complement Activation by Quantitating C3 Deposition on the Yeast Surface*

An enzyme-linked immunoabsorbent assay (ELISA) was developed to measure the amount of C3 deposited on known quantities of yeast during incubation with the 3 human serum preparations used above. Various dilutions of yeast suspensions were first heated to 100°C for 60 min to remove endogenous peroxidase activity and then incubated in 0.1 ml volumes with 0.1 ml of serum for 30 min at 37°C. The yeast was next washed 3 times in 0.05 M phosphate-buffered saline (PBS) containing 0.05% Tween 20. They were then incubated with 0.5 ml of a 1:1000 dilution of peroxidase-conjugated goat antihuman C3 (Cappell Laboratories, Cochranville, Pennsylvania) for 30 min at 37°C and again washed 3 times with PBS-Tween 20. After removal to a fresh test tube, 1.0 ml of substrate was added (1 mg/ml *O*-phenylenediamine, Eastman Kodak Co., Rochester, New York, in 0.05 M citrate buffer, pH 4.5, and 0.05% $H_2O_2$). After 40 min of incubation at room temperature, the supernatants were separated by centrifugation and read in a spectrophotometer at 420 nm. Data were expressed as optical density times 1000 per μg of yeast.

### *Determination of Antigen Content in Yeast Extracts by Inhibition of Antibody Activity*

An ELISA-type antibody assay was set up for determining antibody activity against extracts of *C. albicans* and *P. orbiculare* in normal human serum. Polystyrene tubes were coated with 1.0 ml of appropriate dilutions of either extract in 0.05 M PBS at pH 7.6 for 1 hr at room temperature. The tubes were washed 3 times with PBS-Tween 20, treated with 1% bovine serum albumin, and washed again. Following this step, 0.5 ml of a 1:50 dilution of a pool of serum from 10 normal subjects was added and incubated at 37°C for 30 min. The tubes were washed 3 times with PBS-Tween 20, and 0.5 ml of a 1:200 dilution of peroxidase-labeled, affinity-purified goat antihuman IgG (Litton Bionetics, Kensington, Maryland) was added. After incubation for 30 min at 37°C, the tubes were again washed 3 times with PBS-Tween 20, and 1.0 ml of the substrate described above was added. The tubes were incubated for 40 min and read at 420 nm. Blanks were set up without the human serum and these values subtracted from the experimental values. The experiments were carried out by incubating the pooled human serum with various amounts of 4 extracts each of *C. albicans* and *P. orbiculare* and then testing the serum for residual antibody activity against the corresponding organism. The resulting percent inhibition of the optical density obtained was plotted against the quantity of extract employed (based on protein plus carbohydrate content) to find the amount necessary for 50% inhibition.

Controls for the specificity of the peroxidase-labeled antihuman heavy-chain reagents were carried out in our system as follows: purified immunoglobulins of the 3 classes (Cappell Laboratories, Cochranville, Pennsylvania) were coated onto plastic tubes at a concentration of 10 μg/ml, washed, and incubated with dilutions of the 3 peroxidase-labeled reagents. Good agreement was obtained between the stated specificity and actual antibody activity against the appropriate immunoglobulin class in this test.

### *Human Sera*

Three sera from normal persons and one from an otherwise normal, but C2-deficient person were used for the studies of complement activation. These sera were stored in aliquots at −70°C. The pool of normal sera was prepared using equal volumes of serum from 10 normal subjects without a history of superficial fungal infections. These sera were kept at −20°C. We have previously demonstrated that normal human serum has antibody against *Pityrosporum* as well as *Candida* [8].

*Statistics*

In the complement assays, 4 different yeast preparations representing 2 isolates each of the two organisms were used. For the studies comparing protein and carbohydrate contents of extracts, 6 ether extracts and 4 sonicates were tested, representing 5 isolates of each organism in all. The extracts that were used in the studies of antigen content and complement-activating ability consisted of 2 ether extracts and 2 sonicates representing 2 isolates of each organism. Comparisons were made between the various groups using the unpaired, two-tailed *t*-test.

## RESULTS

### *Complement Activation by* C. albicans *and* P. orbiculare

In Table I, the ability of the two organisms to activate complement is expressed as the percent inhibition of rabbit RBC hemolysis after incubation of 5.0% and 0.5% yeast suspensions with the serum. No significant differences were found between the two organisms at either concentration using normal serum or serum lacking the classical pathway by virtue of calcium chelation or C2 deficiency. Similar results were obtained when the yeasts were quantified after lyophilization of aliquots and weighing (results not shown). It should be noted that hemolysis by C2-deficient serum was affected less by incubation with the two yeasts or with inulin than was normal serum with or without Mg-EGTA. This finding may be related to the mildly deficient function of C2-deficient serum in this system as has been reported previously [9]. As shown in Table II, heating at 56°C for ½ hr significantly suppressed the ability of either normal or C2-deficient serum to cause hemolysis of rabbit RBC.

The ability of extracts of the two organisms to activate complement was also evaluated using the rabbit RBC system.

TABLE I. *Percent inhibition of rabbit RBC hemolysis by human serum complement using constant volumes of yeast*

| Serum | *Candida* (N = 4) | *Pityrosporum* (N = 4) | Statistical significance |
|---|---|---|---|
| *5.0% yeast by volume (mean ± SE of % inhibition)* | | | |
| Normal | 91.0 ± 2.7 | 85.3 ± 2.7 | NS |
| Normal + Mg-EGTA | 83.5 ± 6.1 | 86.8 ± 2.0 | NS |
| C2-deficient | 33.8 ± 10.4 | 49.8 ± 9.0 | NS |
| *0.5% yeast by volume (mean ± SE of % inhibition)* | | | |
| Normal | 30.5 ± 2.1 | 26.8 ± 3.2 | NS |
| Normal + Mg-EGTA | 32.8 ± 11.3 | 34.3 ± 12.1 | NS |
| C2-deficient | 1.0 ± 0.6 | 6.0 ± 1.5 | $p < .02$ |

Note: Inulin at 10 mg/ml produced 94% suppression of hemolysis using normal serum, 95% using normal serum + Mg-EGTA, and 28% using C2-deficient serum.

TABLE II. *Comparison of unheated serum vs. serum heated at 56°C for 30 min in the assays of complement activation*

| Rabbit RBC Hemolysis | | | |
|---|---|---|---|
| Serum | Unheated (% lysis) | Heated (% lysis) | Percent of activity remaining |
| Normal | 74.1% | 1.7% | 2.3% |
| C2-deficient | 58.8% | 2.4% | 4.1% |
| **C3-Deposition on Yeast Surface as Measured by the ELISA Assay** | | | |
| Serum | Unheated (OD × 1000) | Heated (OD × 1000) | Percent of activity remaining |
| *Candida* | | | |
| Normal | 770 | 60 | 7.8% |
| C2-deficient | 470 | 63 | 13.4% |
| *Pityrosporum* | | | |
| Normal | 947 | 138 | 14.6% |
| C2-deficient | 782 | 163 | 20.8% |

These data are from 1 representative experiment of 3 that were performed.

TABLE III. *Percent inhibition of rabbit RBC hemolysis by human serum using 500 μg/ml of yeast extracts*

| Extract[a] | *Candida* | *Pityrosporum* |
|---|---|---|
| *Normal human serum* | | |
| S1 | 22.2[b] | 25.0 |
| S2 | 27.7 | 7.0 |
| E1 | 11.1 | 0.0 |
| E2 | 12.4 | 0.0 |
| *Normal human serum + Mg-EGTA* | | |
| S1 | 26.7 | 20.4 |
| S2 | 38.0 | 7.8 |
| E1 | 2.0 | 5.4 |
| E2 | 2.0 | 22.3 |

[a] Extracts represent 2 sonicates (S1 and S2) and 2 ether extracts (E1 and E2) of each organism.

[b] Data represent % inhibition of hemolysis after incubation with 500 μg/ml of the indicated extract.

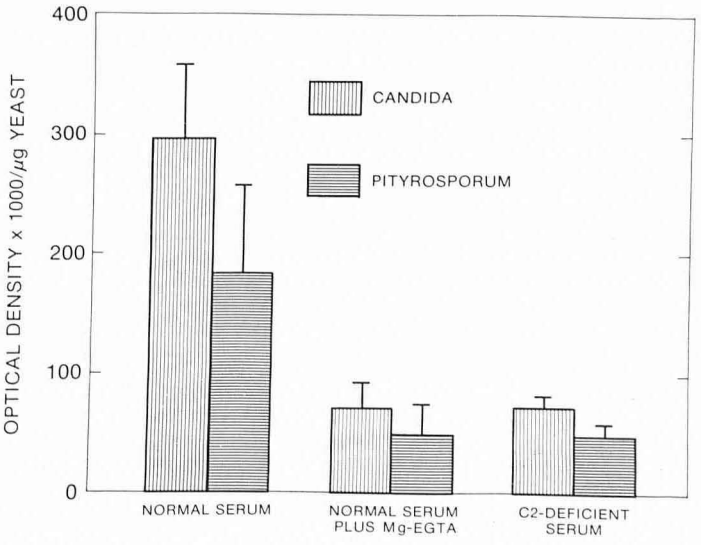


FIG 1. Deposition of C3 on the surface of *C. albicans* and *P. orbiculare* (4 isolates each) using normal human serum, normal human serum plus Mg-EGTA, or C2-deficient human serum. Deposition of C3 was determined using an ELISA system as outlined in Materials and Methods and the results expressed as optical density × 1000/μg of yeast. The differences in C3 deposition were not significantly different for the two types of yeast with any of the 3 types of serum. For *Candida*, but not *Pityrosporum*, there was a significant difference between C3 deposition using normal serum vs. either Mg-EGTA-treated serum or C2-deficient serum ($p < 0.02$ in both cases).

Two ether extracts and 2 sonicates of each organism were tested in this system at a concentration of 500 μg/ml (combined protein and carbohydrate contents), the approximate amount at which whole yeast produced 20% inhibition of hemolysis after incubation with the test serum. As shown in Table III, the results were quite variable, although some complement-activating ability was evident for some of the extracts.

Complement activation was also measured by determining the amount of C3 deposited on the yeast cell surface. This technique was developed because of the possibility that inhibition of rabbit RBC lysis by the yeast could have been due to an actual complement inhibitor of some kind rather than to the consumption of complement components by their activation. As shown in Fig 1, there are no significant differences in the amount of C3 deposited on the surface of the two organisms after incubation with human serum. It would appear that more C3 is deposited on the yeast surface if serum with an intact classical pathway is used. The difference between the results obtained with normal serum vs. that treated with Mg-EGTA or C2-deficient serum was significant for *Candida* ($p < 0.02$ in

TABLE IV. *Protein and carbohydrate contents of* Candida *and* Pityrosporum *extracts*

| Organism | Protein | Carbohydrate | Protein/carbohydrate |
|---|---|---|---|
| Sonicates[a] | | | |
| *Candida* | 9.30± 0.82 | 13.58 ± 3.75 | 0.93 ± 0.34 |
| *Pityrosporum* | 4.71 ± 1.76 | 1.44 ± 0.68 | 3.54 ± 0.43 |
| Statistical significance[b] | $0.1 > p > 0.05$ | $p < 0.02$ | $p < 0.01$ |
| Ether extracts[a] | | | |
| *Candida* | 3.57 ± 1.21 | 5.67 ± 1.30 | 0.80 ± 0.37 |
| *Pityrosporum* | 2.57 ± 0.61 | 0.69 ± 0.13 | 3.94 ± 0.77 |
| Statistical significance[b] | NS | $p < 0.01$ | $p < 0.01$ |

[a] Comparisons were made between protein and carbohydrate contents (expressed as mg/ml of organisms ± S.E.) for 4 sonicates and 6 ether extracts of each organism.

[b] Statistical significance by the unpaired, two-tailed *t*-test was taken at $p < 0.05$.

TABLE V. *Antigenic activity of* Candida *and* Pityrosporum *extracts in an ELISA assay*

| Organism | μg of Extract for 50% inhibition[a] | $Log_{10}$ values |
|---|---|---|
| *Candida* | 70.0 ± 28.1 | 1.73 ± 0.2 |
| *Pityrosporum* | 822.6 ± 260.4 | 2.83 ± 0.2 |
| Statistical significance[b] | $p < 0.05$ | $p < 0.01$ |

[a] Combined protein and carbohydrate contents for 2 ether extracts and 2 sonicates of each organism required for 50% inhibition of antibody activity (± S.E.).

[b] Statistical significance by the unpaired, two-tailed *t*-test.

both cases) but not for *Pityrosporum*, perhaps because of greater variability in the results obtained with the latter.

As shown in Table II, heating serum to 56°C for ½ hr significantly reduced the amount of C3 deposited on the surface of both yeasts using either normal or C2-deficient serum.

### *Protein, Carbohydrate, and Antigenic Contents of* Candida *and* Pityrosporum *Extracts*

As shown in Table IV, extracts of both organisms contained comparable protein contents, whereas those of *Candida* contained significantly more carbohydrate. This factor resulted in lower protein to carbohydrate ratios for extracts of *Candida* than for those of *Pityrosporum*. Antigenic contents of the extracts were measured using inhibition of antibody activity of pooled human serum against the organisms in an ELISA system as described under Materials and Methods. The results demonstrated that *Candida* appears to have a significantly higher content of antigen than does *Pityrosporum* (Table V), a finding compatible with our previous studies using the lymphocyte-transformation system [5].

## DISCUSSION

These experiments indicate that the two yeasts, *C. albicans* and *P. orbiculare*, appear to have approximately equal abilities to activate complement using either the rabbit RBC hemolytic system to measure alternative pathway activity left in human serum after incubation with the organisms or measurement of C3 deposition on the yeast surface using an ELISA system. Complement-activating ability appeared to be comparable for the two organisms in both systems using either normal human serum or serum with a nonfunctional classical pathway because of C2 deficiency or the addition of Mg-EGTA. In addition, these studies have demonstrated that extracts of *Candida* contain more carbohydrate and antigenic activity than do those of *Pityrosporum*.

Many microorganisms that are pathogenic for humans initiate complement activation via the alternative pathway. These include: (a) other *Candida* species in addition to *C. albicans* [10]; (b) *Cryptococcus neoformans* [11]; (c) *Paracoccidioides brasiliensis* [12]; (d) *Streptococcus pneumonia* [13]; and (e) the sindbis virus [14]. In addition, complement activation has been suggested as a possible cause of inflammation in acne [15]. Finally, complement activation, particularly that occurring via the alternative pathway, has been demonstrated to be important in the defense against experimental intravascular infections with *C. albicans* [16] and pneumococci [17]. Whether or not this process is really important in the host defense against these pathogens causing human disease is unclear. This situation is comparable to the status of alternative pathway activation by small molecules as a cause of some cases of asthma—an attractive hypothesis but without solid evidence backing it [18].

The results of the present investigation indicate that the lack of inflammation in the lesions of tinea versicolor is not due to the inability of the causative organism, *P. orbiculare*, to activate complement. This organism is approximately as capable in initiating complement activation by both pathways as is *C. albicans*, a producer of highly inflammatory cutaneous lesions. This finding is interesting inasmuch as *Pityrosporum* appears to contain significantly less extractable carbohydrate and antigenic material than does *Candida*. The ability to activate complement is obviously not a property singularly sufficient for an infecting organism to produce inflammatory skin lesions. Although in biopsies of *Candida*- and *Pityrosporum*-infected skin in humans the organisms are generally found at the same locations, i.e., confined to the stratum corneum [2,19], it is possible that a greater capacity of *Candida* for deeper invasion through the stratum corneum may be the reason for the differences in inflammation produced by the two organisms. Evidence to support such an explanation has been generated in studies of experimental cutaneous infections of mice produced by *C. albicans* and other *Candida* species [3]. In addition, it is possible that *Pityrosporum* does not release soluble complement-activating factors that can diffuse across the stratum corneum to encounter a source of activatable complement. However, an alternative explanation is that some factor instead of or in addition to complement activation is important for the production of inflammation in superficial fungal infections. Differences in the quantity of antigen present and the ability to elicit immunologic reactions could account for the differences in inflammatory potential of these two organisms, as we have suggested previously [5].

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# Antizyme Release Is an Early Event in Ornithine Decarboxylase Induction by Hair Plucking*

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**Plucking of hair from the dorsal skin of rats resulted in a rapid decrease in ornithine decarboxylase (ODC) activity. A significant loss of activity did not occur in other skin enzymes under the same conditions and in vivo incorporation of [$^3$H]-leucine in skin was not significantly decreased 60 min immediately following hair plucking. Treatment of ODC enzyme preparations with 10% $(NH_4)_2SO_4$ resulted in recovery of approximately 75% greater ODC activity than in untreated samples, suggesting the presence of an inhibitor (antizyme). ODC inhibitor was detected in plucked skin; inhibitor levels increased after treatment of plucked skin extracts with 10% $(NH_4)_2SO_4$.**

In the rat, hair growth occurs in a cyclic manner. Plucking of hair during the telogen phase induces high levels of ornithine decarboxylase (L-ornithine carboxylase, EC 4.1.1.17) (ODC) [1] within 4 hr [2]. However, during the 60 min immediately following hair plucking ODC activity is observed to decrease [1,3]. The cause of this initial loss of ODC enzyme activity is the subject of this report.

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Abbreviations:
DDT: dithiothreitol
EDTA: (ethylenedinitrilo)-tetraacetic acid, disodium salt
ODC: ornithine decarboxylase
PLP: pyridoxal-5′-phosphate
TCA: trichloroacetic acid
Tris: trihydroxyaminomethane

## MATERIALS AND METHODS

### *Chemicals*

Trihydroxyaminomethane (Tris), pyridoxal-5$^1$-phosphate (PLP), and cycloheximide, were purchased from Sigma Chemical Co., St. Louis, Missouri. DL-[1-$^{14}$C]-ornithine (51.2 mC/mmol), L-[4,5-$^3$H]-leucine (12 Ci/mmol), [1,4-$^{14}$C]-putrecine (89.9 mCi/mmol), phenethylamine, and Aquasol II were from New England Nuclear, Boston, Massachusetts. Enzyme-grade sucrose and ultrapure ammonium sulfate were from Schwartz-Mann, Spring Valley, New York. Dithiothreitol (DTT) was from Bachem Feinchemikalien, Liestal, Switzerland. Biogel-P100 was obtained from Bio-Rad Laboratories, Richmond, California.

### *Animals*

Juvenile male CD strain rats weighing 80–100 g (24–28 days old) were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. For ODC enzyme studies the animals were used in the early anagen phase of hair growth, which was defined as dorsal skin for which the most ventral edge of a patch was within 1 cm of the discernible border of new hair eruption. For inhibitor studies, animals in the telogen phase were used. This phase was defined as skin for which no border of new hair growth was detected on the animal's dorsum.

### *Time Course of Plucking Response*

Animals were anesthesized with ether and the hair plucked from their dorsal skin in patches 60, 45, 30, 15, 5, and 0 (immediately) min before death by decapitation. Hair plucking was accomplished using hemostat forceps which had thin-walled rubber tubing forced over the ends. The hair could then be grasped tightly and jerked free in clumps. A 2.5 × 2.5 cm skin patch can be plucked bare in 15–20 sec by this method. The skin patches were excised, cleaned of underlying musculature, weighed, and homogenized 1:9 (weight:volume) in ODC buffer (10 mM Tris pH 7.0, 0.5 mM EDTA pH 7.0, 0.01 mM PLP, and 5 mM DTT) as previously described [2]. ODC assays and turbidimetric protein determinations were performed as previously described [3]. One unit of ODC activity is capable of releasing 1 nmol $^{14}CO_2$/hr.

### *Response of Other Skin Enzymes to Hair Plucking*

Animals were anesthesized with ether and the hair plucked from one half of their dorsal skin 60 and 0 min before death by decapitation. The